

DEPARTMENT OF HEALTH AND HUMAN SERVICES

NOTE TO THE FILE

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**Subject:** Canola which expresses a phytase gene derived from *Aspergillus niger*

Keywords:

Canola (*Brassica napus*), *Aspergillus niger* phytase gene, myo-inositol-hexakisphosphate-3-phosphohydrolase (3-phytase), kanamycin resistance (nptII) gene, neomycin phosphotransferase II (NPTII protein), Phytaseed®

Background

In submissions dated October 7, 1997 and January 21, 1999, the law firm of Morgan, Lewis & Bockius, representing BASF AG, provided summary information to support the safety and nutritional assessment of the transgenic phytase-producing canola lines, MPS961, MPS962, MPS963, MPS964, and MPS965. Canola includes varieties of oilseed rape with defined characteristics related to their content of two toxicants, erucic acid and the glucosinolates.

Intended Effect and Food/Feed Use

According to the developer, the intended technical effect of the genetic modification of canola is to allow the plant to produce a fungal 3-phytase. This enzyme can be utilized to increase the breakdown of plant phytates which bind phosphorus. Phytate is the major storage form of phosphorus in many seeds and phytate-bound phosphorus is unavailable to monogastric animals. Since monogastric animals are not able to degrade this molecule, much of the phosphorus bound to phytate passes into the environment through the manure. Use of the enzyme and appropriate management techniques can lead to a reduction in the phosphorus content of manure thus, improving environmental conditions.

The phytase-containing canola will be produced under contract by growers with the harvested seed being double flaked to render it non-viable. After flaking, seed viability and phytase concentrations will be determined. The phytase activity content of the flaked seed will be standardized by addition of microbial phytase or nontransgenic canola seed. The standardized flaked seed (having the trade name Phytaseed®) will be directly incorporated in animal feed as a source of the enzyme.

The submission states that the phytase gene was obtained from *Aspergillus niger* var. van Tieghem. The expression product, 3-phytase, has an International Union of Biochemistry (IUB) number of EC 3.1.3.8, while its Chemical Abstract Service Registry (CAS) number is 37288-11-2. The nptII gene was isolated from transposon Tn5 in *Klebsiella pneumonia*. BASF indicates that this type of transposon can be found in many bacterial species. The nptII protein, neomycin phosphotransferase II, confers resistance to some aminoglycoside antibiotics including neomycin and kanamycin, and was used by the firm as a selectable marker for transformed plant cells.

BASF reports that *Brassica napus* has a long history of human usage with commercial plantings of oilseed rape for industrial purposes occurring in the Netherlands during the 16th century. BASF also reports that in Asia the oil was considered edible. In the 1940s the firm states that traditional plant breeding techniques led to improved qualities in oilseed rape. These improvements in meal and oil quality led to dramatic increases in rapeseed production both in Europe and North America. The firm reports that 2 species, *rapa* and *napus*, have the capability to produce high quality food products which contain low levels of erucic acid in the oil and small amounts of the glucosinolates in the extracted meal. The oil derived from canola or these improved oilseed rape varieties, is utilized in human foods, while the meal is used as an animal feedstuff. BASF also notes that up to four genetically modified rapeseed varieties have been accepted for commercial introduction in several national markets.

#### Molecular Alterations and Characterization

The novel genetic material in the new canola lines was inserted into the canola variety, Westar, using *Agrobacterium tumefaciens*-mediated transformation with the disarmed Ti-plasmid, pMOG625. The T-DNA contained both phytase and nptII genes. The phytase gene is under the control of the cruciferin A seed storage protein transcript promoter which includes a cruciferin signal peptide sequence. Its terminator is also from the cruciferin A seed storage protein transcript. Both controlling sequences were obtained from *Brassica napus*. The nptII gene is under the control of the NOS promotor and terminator with an *Agrobacterium tumefaciens*-derived open reading frame inserted between the gene and its terminator. The open reading frame consists of coding for 50 amino acids from the *Agrobacterium* ornithine-cyclo-deaminase. The firm states that if expression occurs, the peptide is unlikely to be active as it consists of only 14% of the original enzyme. The nptII gene was used as a selectable marker to identify primary transformant plants. This gene originated from transposon Tn5 from *Klebsiella pneumonia*. BASF indicates that this type of transposon can be found in many bacterial species. The original primary transformants were selected on kanamycin-containing media.

Transgenic plants were derived from one primary transformant which had a phytase expression level of 315 phytase units/ g. (One phytase unit (FTU) is the amount of enzyme which liberates 1 micromole inorganic phosphorus per minute from sodium phytate, 0.0051 moles/ liter, at 37°C and at pH 5.0). These plants were selected based on Southern analyses indicating that they had either 1 or 2 integration points. The plants were then used to generate the homozygous lines: MPS961, MPS962, MPS963, MPS964, and MPS965. These lines have either 1 or 2 copies of the phytase gene as determined by PCR analysis. BASF reports that the entire plasmid, pMOG625, was inserted into the canola genome in line MPS965. Lines MPS961, MPS962, MPS963, and MPS964 do not carry the bacterial selection marker nptII, nor the bacterial origin of replication as demonstrated by Southern analyses. These lines do contain varying amounts of the transformation plasmid beyond its right and left borders areas. BASF states that the modified lines are genetically stable as the gene is expressed in the progeny.

### Expressed Proteins/ Regulatory Considerations

The firm reports that expression of the phytase gene can lead to enzyme levels of 60 to 600 FTU/g seed. No expression of this gene is reported to occur outside of the seed. The expressed phytase protein is an 85 KD glycopeptide and catalyzes the conversion of plant phytate to inositol and inorganic phosphate thus, making plant phytate-bound phosphorus available to monogastric species. Expression levels of phytase, the desired product, ranged from 57.8 to 91.3 FTU/ g in the modified seed. In contrast, the parent, Westar, contained less than 8 FTU/ g. The firm indicates that the inserted gene codes for a protein which is equivalent in terms of amino acid composition to the wild type *Aspergillus niger* phytase gene-encoded enzyme, which is used to produce an enzyme product currently used in animal feed.

Only MPS965 of the transgenic lines contains an expressed neomycin phosphotransferase II gene. Expression levels of nptII have not been quantified. Neomycin phosphotransferase II, also known as aminoglycoside 3'-phospho-transferase II, is an approved food additive under 21 CFR 173.150 and 573.130 for use as a processing aid in the development of new varieties of tomato, oilseed rape, and cotton.

The developer indicates that none of the introduced genes or their expression products in the transgenic canola has a history of toxicity or allergenic concern based on extensive literature surveys. The firm does indicate that inhalation of enzymes can cause allergenic sensitization, but that the risk for this occurring with the transgenic canola is negligible. BASF concludes that the genetically modified rapeseed should not be considered more allergenic than non-transgenic counterparts.

### Compositional Assessment

The developer indicates that the seed will be fed to monogastric species as a source of the phytase protein. Analytical measures for flaked seed included seed phytase activity, oil and carbohydrate content, and its fatty acid profile. Milled seed was analyzed for moisture, crude protein, crude fat, crude fiber, ash, calcium, phosphorus and fatty acid levels. The firm also determined levels of erucic acid and the glucosinolates in whole and ground seed. The developer reports that the Phytaseed® producing lines maintained the canola qualities of the parent in that the levels of the toxicants, erucic acid and the glucosinolates, remained low and did not differ substantially from the parent variety. The genetic modification also did not significantly alter levels of the seed's macro-constituents, oil and carbohydrates. Overall, BASF concludes that there are no substantive differences between the Phytaseed® producing lines and control canola except for the intended effect of the presence of the phytase enzyme.

The firm conducted one trial each in broiler and laying chickens to demonstrate enzyme effectiveness. The firm also presented the analytical methodology used to determine phytase concentrations. Information from several toxicity trials conducted with the fungally derived enzyme to assess product safety was reported. These trials included acute oral and inhalation toxicity tests in rats, acute dermal and ocular irritation trials in rabbits, microbial and mammalian mutagenic/clastogenic studies, and 14-day and 90-day subacute oral toxicity trials in rats. The firm also reported results from a broiler study conducted using Phytaseed® canola, which included some toxicological end-points.

BASF has concluded that the genetically modified lines are substantially equivalent to both rapeseed and a phytase enzyme derived from *Aspergillus niger*. The firm reports that the enzyme obtained from *Aspergillus niger* has been extensively tested and has been found to be nontoxic and safe for use in animal feed.

### Conclusions

BASF has concluded based on its safety and compositional assessment, that canola modified to produce 3-phytase is not materially different in composition, safety, or any relevant parameter except for the intended effect from canola now grown, marketed, and consumed. At this time, based on the firm's description of the data and analysis, the Agency considers the consultation on Phytaseed® producing canola lines, MPS961, MPS962, MPS963, MPS964, and MPS965, to be complete.

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